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Award Number: DAMD17-00-1-0074

TITLE: p16 Axis in Androgen-Dependent Proliferation of Prostate Cancer Cells

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REPORT DATE: April 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2003	Annual (1 Apr 02 - 31 Mar 03)

4. TITLE AND SUBTITLE
p16 Axis in Androgen-Dependent Proliferation of Prostate Cancer Cells

5. FUNDING NUMBERS
DAMD17-00-1-0074

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The purpose of this study is to understand the role of the p16 growth control axis in androgen dependent proliferation of prostate cancer cells. The p16 axis contains two tumor suppressors (p16Ink4a and RB), cyclin D-dependent kinases, and transcription factor E2F. We hypothesized that functions of the p16 axis can influence androgen-dependence of prostate cancer cells. To test this hypothesis, we proposed to use controlled expression techniques to determine whether disruption of p16 axis function can lead to androgen-independence in human androgen-dependent prostate cancer cells (LNCaP) and, on the other hand, whether restoration of p16 axis function can restore androgen-dependence in androgen-independent prostate cancer cells (DU-145). We have now established LNCaP derivative cell lines that can provide inducible expression of exogenously introduced proteins. In DU-145 cells, we have discovered that restoration of protein expression of pRB and the androgen receptor cause cell death. This finding reveals a novel functional relationship between RB and the AR in prostate cancer biology and may provide potential strategy for prostate cancer gene therapy.

14. SUBJECT TERMS
androgen dependence, cell cycle regulation, tumor suppressors, androgen receptor, inducible expression cell lines, cell death

15. NUMBER OF PAGES
14

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

The subject of this proposal is the role of the p16 growth control axis in androgen dependent proliferation of prostate cancer cells. The p16 axis contains two tumor suppressers (p16Ink4a and pRB), an important component of the cell cycle machinery (cyclin D-dependent kinases), and transcription factor E2F (regulators of gene expression). We hypothesized that functions of the p16 axis can influence androgen regulation of prostate cancer cells. To test this hypothesis, we proposed to use controlled expression techniques to determine whether disruption of p16 axis function can lead to androgen-independence in androgen-dependent prostate cancer cells and, on the other hand, whether restoration of p16 axis function can restore androgen-dependence in androgen-independent prostate cancer cells. We expected that this study will reveal connections between androgen regulation of prostate cancer cells and functions of a central cell growth control pathway. To date, a major outcome of our study is the identification of a novel apoptosis-inducing activity of the tumor suppressor pRB that is specifically dependent on the transactivation activity of the androgen receptor (AR).

Body

The two approved aims in this project are discussed separately below.

Aim 1. To determine whether deregulated expression of positive-acting cell cycle regulators can abrogate the dependence of LNCaP cells on steroid hormones in culture.

The key step in this Aim is to establish LNCaP derivative cell lines that allow controlled inducible expression of various cell cycle regulators. The tetracycline-controlled inducible expression system is a two-step process. The first step is to establish a derivative cell line that expresses the tetracycline-controlled transactivator (tTA). From this tTA cell line, a second derivative line is then established that contains the gene under study that is under the control of the tTA-controlled promoter. We have had experienced difficulties from the poor ability of LNCaP cells to form single colonies as well as to expand from such single colonies in culture. In the last report, we have established LNCaP tTA cell lines through a technical adjustment using retroviral vectors. We then employed the standard transfection approach to generate the second cell line with inducible expression of the E2F transcription factor. Again, we experienced many unsuccessful attempts at this stage. However, we are continuing this line of study by using the retrovirus approach.

LNCaP cells contain functional RB and the AR. In the mean time, we have determined whether the AR and RB in LNCaP cells have any functional relationship, based on our identification of an apoptosis-inducing activity of pRB that is entirely dependent on the AR (see below for Aim 2). For this purose, we have first determine the proteins levels of RB and the AR in response to androgen DHT. We have found that protein levels of the AR decreased dramatically when LNCaP cells were switched from regular media to media containing charcoal dextran treated serum (CDT media). AR protein levels recovered when the cells were subsequently incubated with the supplemented DHT at 1 nM. These findings suggested to us that the previously observed phenotypic responses of LNCaP cells to androgen deprivation could result from the lack of the androgen receptor, which would complicate the studies of the roles of cell cycle regulators in this response. Therefore, we have decided to investigate whether prevention of AR down regulation would have any effects on the androgen response of LNCaP cells. To achieve this, we are in the process of introducing the AR gene under control of the CMV promoter through stable retrovirus infection of LNCaP cells. Once we established LNCaP derivative cell lines that do not down regulate AR expression in response to androgen withdrawal, we will determine whether these cells have the same cell proliferation response to androgen.

Aim 2. To determine whether correction of p16 axis function and androgen receptor expression can restore androgen dependence in DU-145 cells.

The major outcome from studies in this Aim is the identification of a novel apoptosis-inducing activity of RB through the combined re-expression of RB and AR in DU-145 cells. A manuscript describing this work is being submitted for publication. The key results of the manuscript are presented below.

Re-expression of Bax, RB and the AR in prostate cancer DU-145 cells

We used the prostate cancer cell model DU-145, which lacks functional RB and AR, among other mutations, to re-express RB and the AR to study the functional relationships between these two regulators. Although previous reports showed that DU-145 cell lines with constitutive re-expression of RB could be established (suggesting that RB did not block cell cycle progression in DU-145 cells) (Bookstein et al., 1990), we still used inducible expression to re-express RB to create, without and with induction, respectively, a pair of non-RB-expressing and RB-expressing DU-145 cells. Inducible expression avoids possible selection for cells that contain mutations that abolish the inhibitory effects of RB during the generation of derivative cell lines.

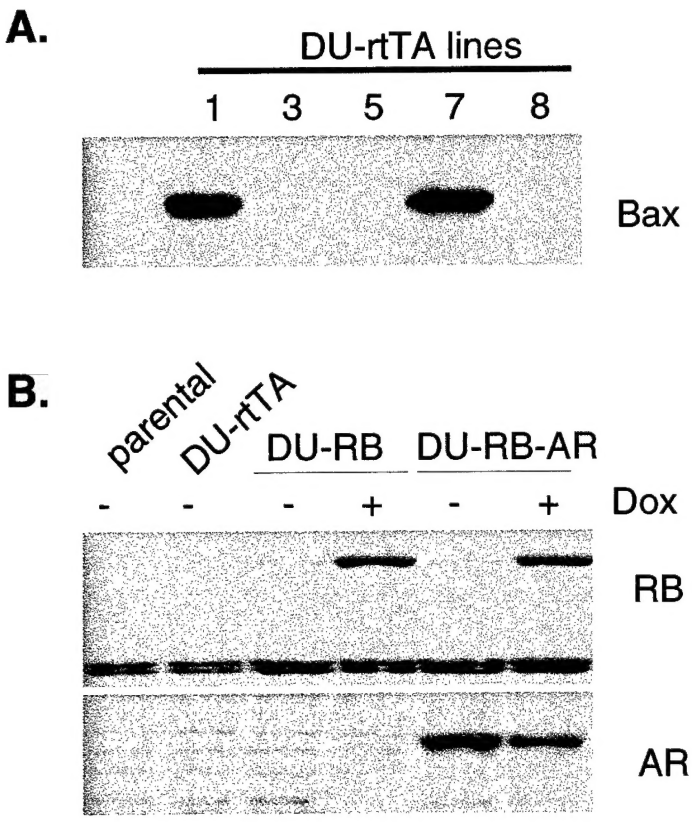

Figure 1. Restoration of Bax, RB, and AR expression in DU-145 prostate cancer cells. **A**. Total cell extracts from parental DU-145 and independent DU-rtTA clonal lines were Western blotted with an anti-Bax antibody. **B**. Total cell extracts from parental DU-145, DU-rtTA, DU-RB, and DU-RB-AR cell lines were Western blotted with antibodies against RB and the AR. RB expression was induced by the addition of Dox in the culture media for 16 hours.

DU-145 cells are microsatellite mutator phenotype positive (MMP+) (Rampino et al., 1997). Defects in DNA mismatch repair (MMR) in MMP+ cells render them prone to replication errors. The human Bax gene contains a sequence of eight consecutive deoxyguanosines (the G-8 track) near its N-terminus, which is prone to mutations due to DNA slippage during DNA replication. It was reported that the G-8 track of the Bax gene is homozygously mutated to G-9 and consequently the Bax protein is not expressed in DU-145 cells (Rampino et al., 1997). In theory, the instability at the 8-G track should generate both wild type-to-mutant and mutant-to-wild type changes if no selective pressure is present to select against Bax-expressing cells. We therefore wished to determine whether we could restore Bax expression during the generation of various cell clones so that the functional effects of RB and the AR would not be affected by the absence of Bax, which has been shown to be required for certain types of apoptosis in certain tumor cell lines.

We transfected DU-145 cells with pUHD-172neo (encoding a reverse tetracycline regulated transactivator and the G418 resistant gene), established clonal lines with G418 selection, and screened for the clonal lines for their ability to express a test gene in response to Dox. Five such clones were found to have this ability (data not shown). We then determined Bax expression in these clones. As shown in Figure 1A, 2 out of the 5 clones have restored Bax expression. We did not detect any difference in the proliferation rates among all these clones compared with the parental cells (data not shown). We picked clone #1 (the DU-rtTA cells) to establish subsequent cell lines and continued to monitor Bax expression. Bax expression was retained in all the subsequent clones in this study (data not shown).

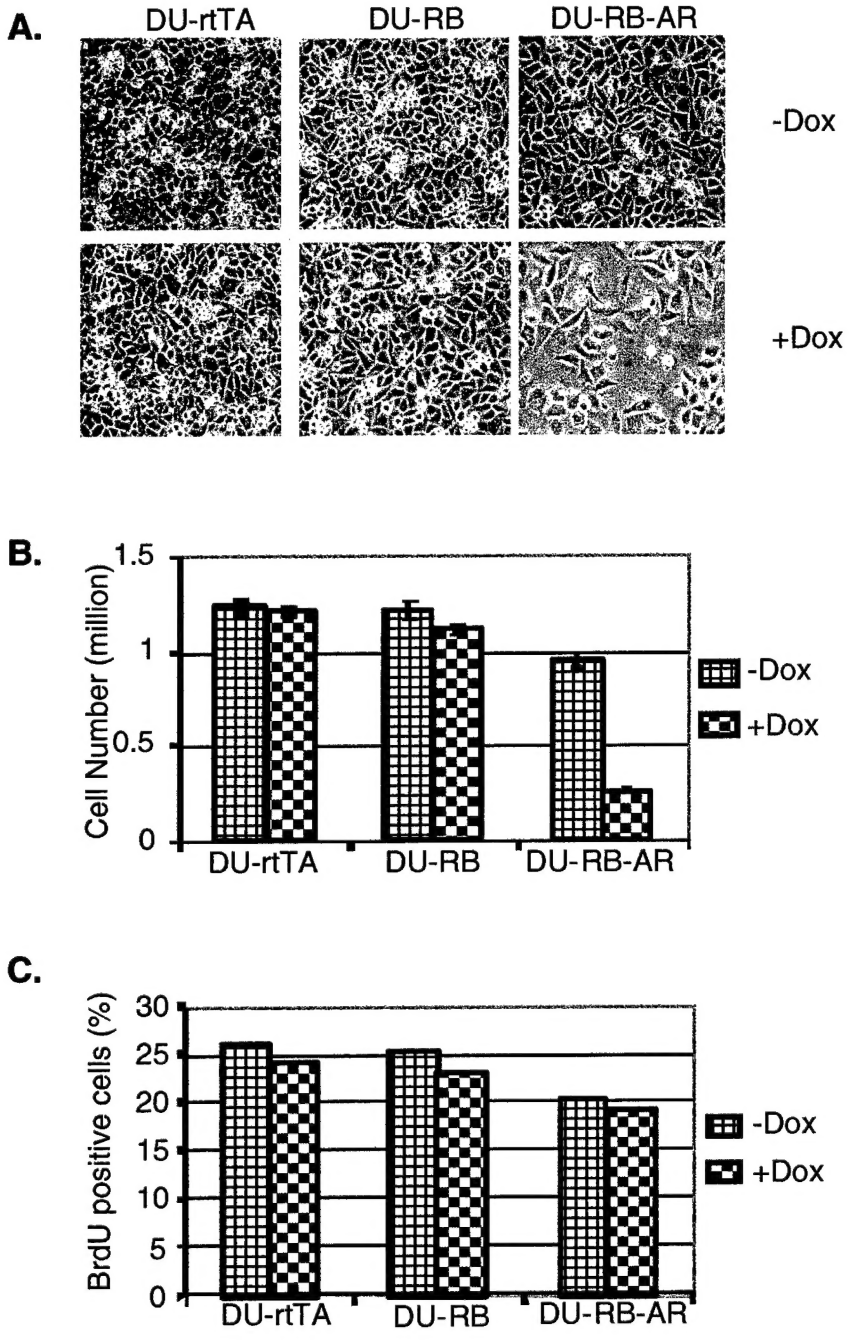

Figure 2. Combined re-expression of RB and the AR in DU-145 cells resulted in reduction in cell numbers but not in BrdU labeling. DU-rtTA, DU-RB, and DU-RB-AR cells were treated with Dox, or untreated in parallel, as indicated, for twenty-four hours. Cells in plates were then photographed under phase-contrast microscope (**A**); total numbers of attached cells were counted (**B**); cells were pulse-labeled with BrdU for 4 hours, fixed, stained with anti-BrdU, and the number of BrdU positive cells determined (**C**).

We then transfected the DU-rtTA cells with pUHD10-3-RB, which we previously used to establish Rb inducible re-expression in osteosarcoma cell Saos-2 (Jiang et al., 2000) and pBabe-puro and selected for puromycin

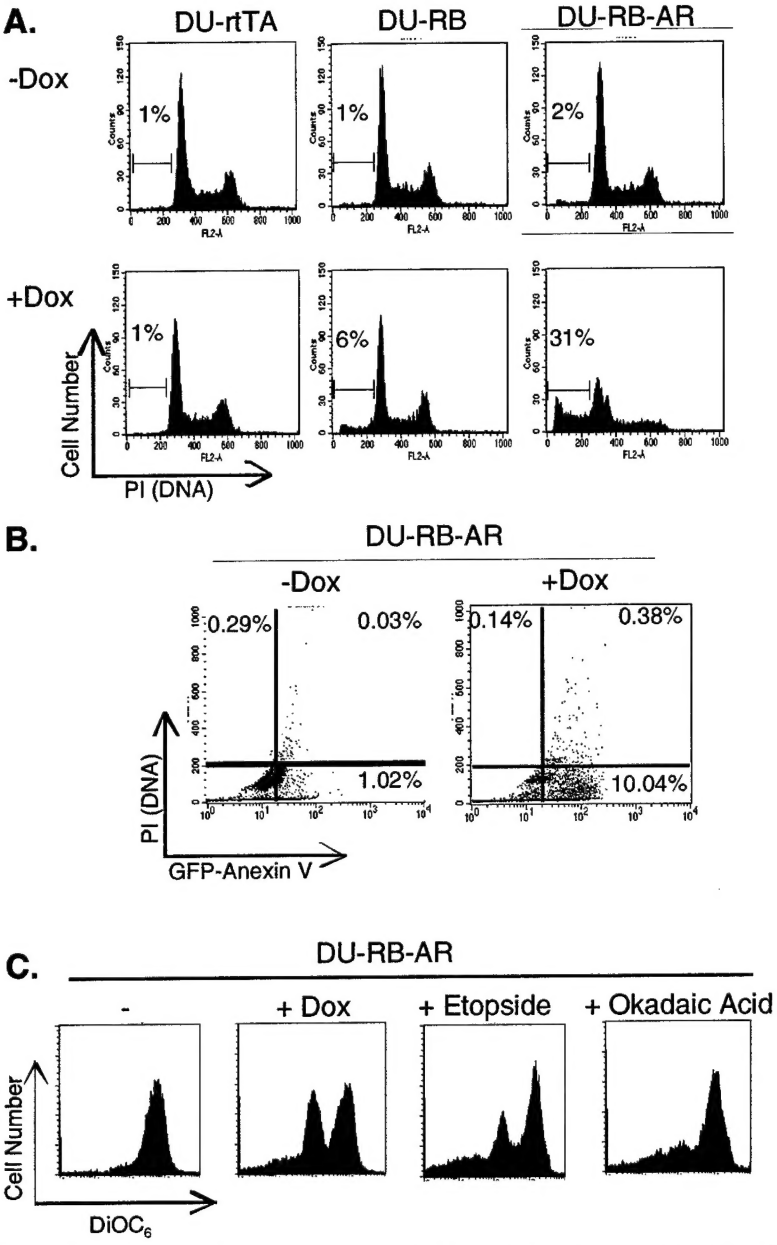

Figure 3. Combined re-expression of RB and the AR in DU-145 cells resulted in apoptosis and mitochondria damage. **A**. DU-rtTA, DU-RB, and DU-RB-AR cells were treated with Dox, or untreated in parallel, as indicated, for twenty-four hours. Total cells (including the floating cells) were fixed in EtOH, stained with propidium iodide (PI), and analyzed by flow cytometry. Percentages of cells in the Sub-G1 region were determined and indicated. **B**. DU-RB-AR cells were similarly induced with Dox, total cells harvested, stained with GFP-Annexin V and PI without EtOH fixation, and analyzed by flow cytometry. Cells in the lower right window were defined as apoptotic since they were Annexin V staining positive but PI staining negative (PI did not leak through the cell membrane). Cells in the upper left window were defined as death through necrosis. Cells appeared in the upper right window in the late stages of apoptosis due to secondary cell membrane damage. **C**. DU-RB-AR cells were similarly induced with Dox, or treated with the indicated apoptosis-inducing chemical without Dox, as indicated, for twenty four hours. Total cells were then labeled with $DiOC_6$ for thirty minutes and analyzed by flow cytometry. Cells in the low-brightness peak contains damaged mitochondria.

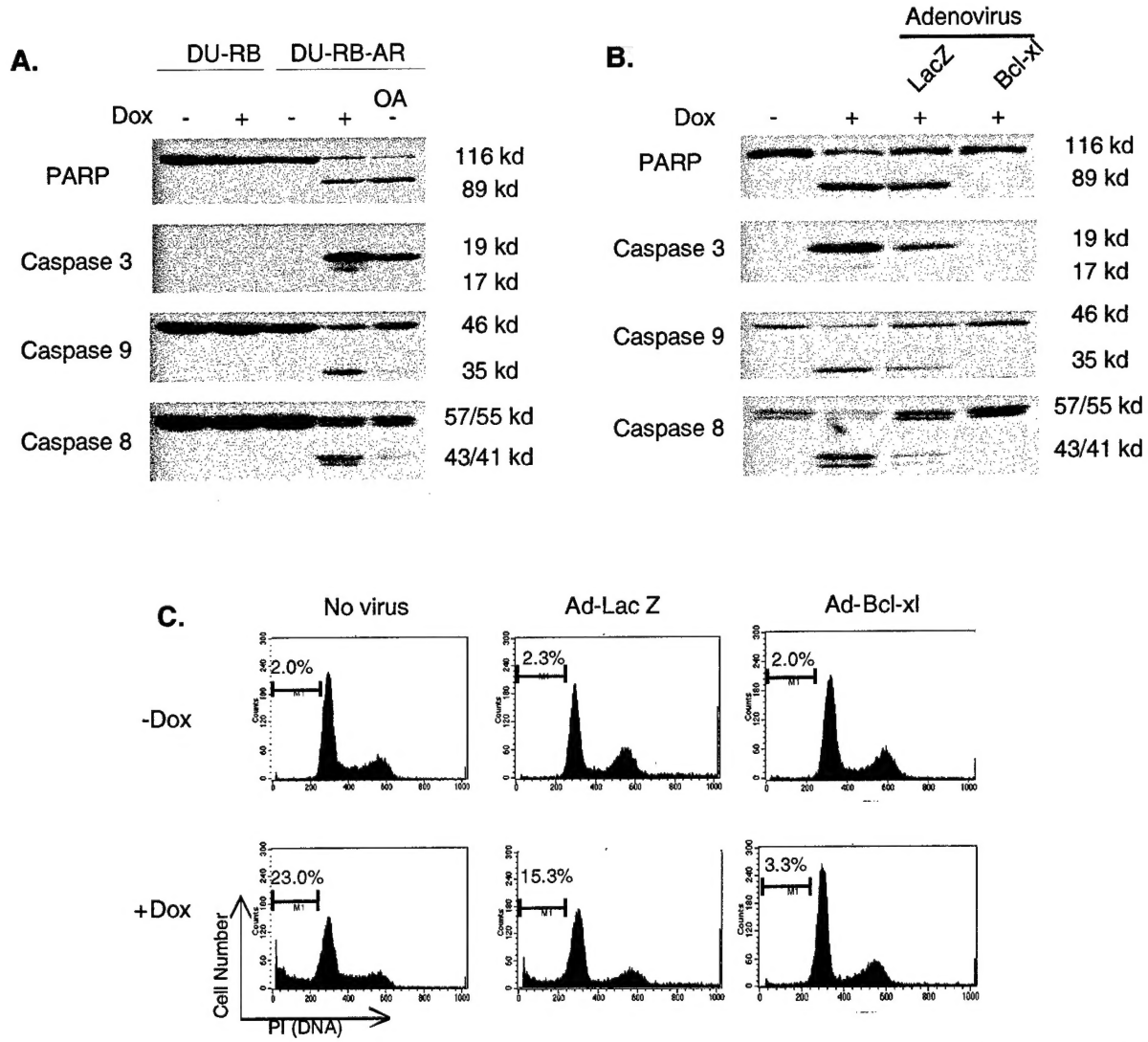

Figure 4. Combined re-expression of RB and the AR in DU-145 cells led to cleavage of multiple cellular caspases, which was prevented by over-expressing Bcl-xl. **A.** DU-RB and DU-RA-AR cells were treated with Dox, or okadaic acid in the absence of Dox, as indicated, for twenty four hours. Cell extracts were made from total cells and analyzed by western blotting with the indicated antibodies. Molecular weights of full-length and cleaved caspases are marked. **B.** DU-RB-AR cells were first infected with adenoviruses expressing either LacZ or Bcl-xl for twenty four hours and then induced with Dox for another twenty four hours. Western blotting were performed as in A. **C.** DU-RB-AR cells were treated as in B, harvested and analyzed for Sub-G1 fraction as in Figure 3A.

resistant clones that could be induced to express RB with Dox. A total of 4 independent cell lines were established and were found to exhibit similar properties. Data from a representative one, DU-RB, (Figure 1B) was chosen for presentation in this report.

From the RB inducible DU145 cells, we further derived lines that contained constitutive expression of the AR to create, without and with induction, respectively, a pair of DU-145 cells that only re-express AR and that re-express both the AR and RB. We transfected DU-RB cells with pcDNA3-ARzeo and selected Zeocin-resistant clones that contained constitutive expression of the AR in the absence of Dox. AR-expressing clones were then confirmed of their ability to re-

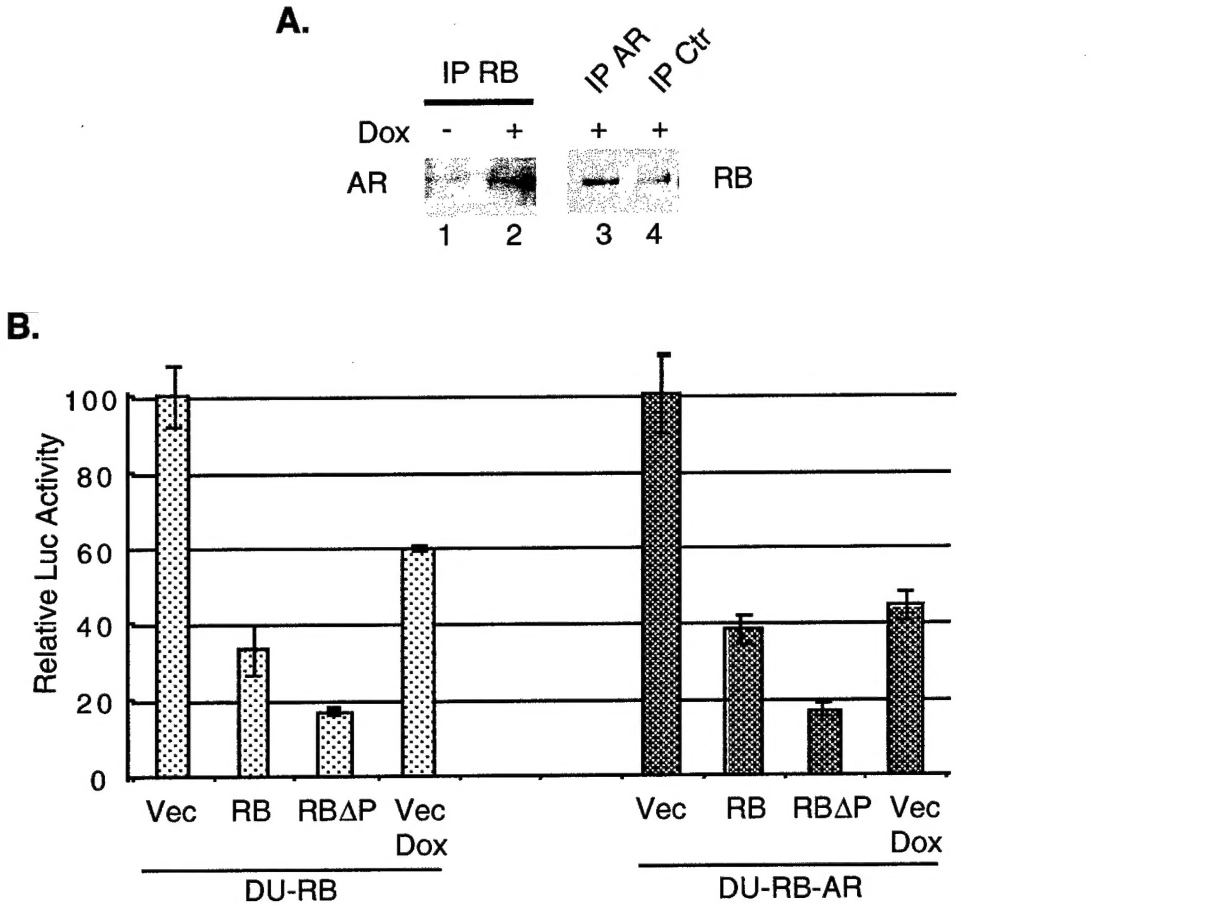

Figure 5. The AR physically interacted with RB but did not alter RB's ability to repress E2F in DU-145 cells. **A.** DU-RB-AR cells were induced with Dox as indicated; total cell extracts were immuno-precipitated with a mouse anti-RB antibody (lanes 1 and 2), or a rabbit anti-AR antibody (lane 3), or a control antibody (normal rabbit IgG, lane 4); and the immuno-precipitates were analyzed by Western blotting with anti-AR (lanes 1 and 2) or anti-RB (lanes 3 and 4). **B.** DU-RB and DU-RB-AR cells were transfected with an E2F reporter plasmid (E2F-TK-Luc) together with the indicated expression vectors. RBΔP is an unphosphorylable RB with phosphorylation sites mutated. Dox, where indicated, was added to the cells for the last seven hours before harvest. DHT was added to all the samples for the last twenty four hours. Luciferase activity was measured and presented as relative units. Triplicate samples were analyzed for every data point and standard deviations displayed as error bars. The experiments were repeated three times with similar results.

express RB in response to Dox. Three separate clones were isolated and one representative clone, DU-RB-AR, was presented in this report (Figure 1B).

Combined re-expression of RB and the AR induces mitochondria-dependent apoptosis

Re-expression of RB did not result in any difference in cell proliferation as measured by cell morphology, cell number determination, and BrdU labeling in induced cells compared with parallel uninduced cells (Figure 2A, B, C). In fact, induced cells could be expanded similarly as uninduced cells. indicating that generation of DU-145 derivative cell lines with constitutive re-expression of RB as reported in previous studies (Bookstein et al., 1990) could indeed be achieved without the selection of mutations that disrupt RB's cell cycle arresting activity. DU-145 cells with

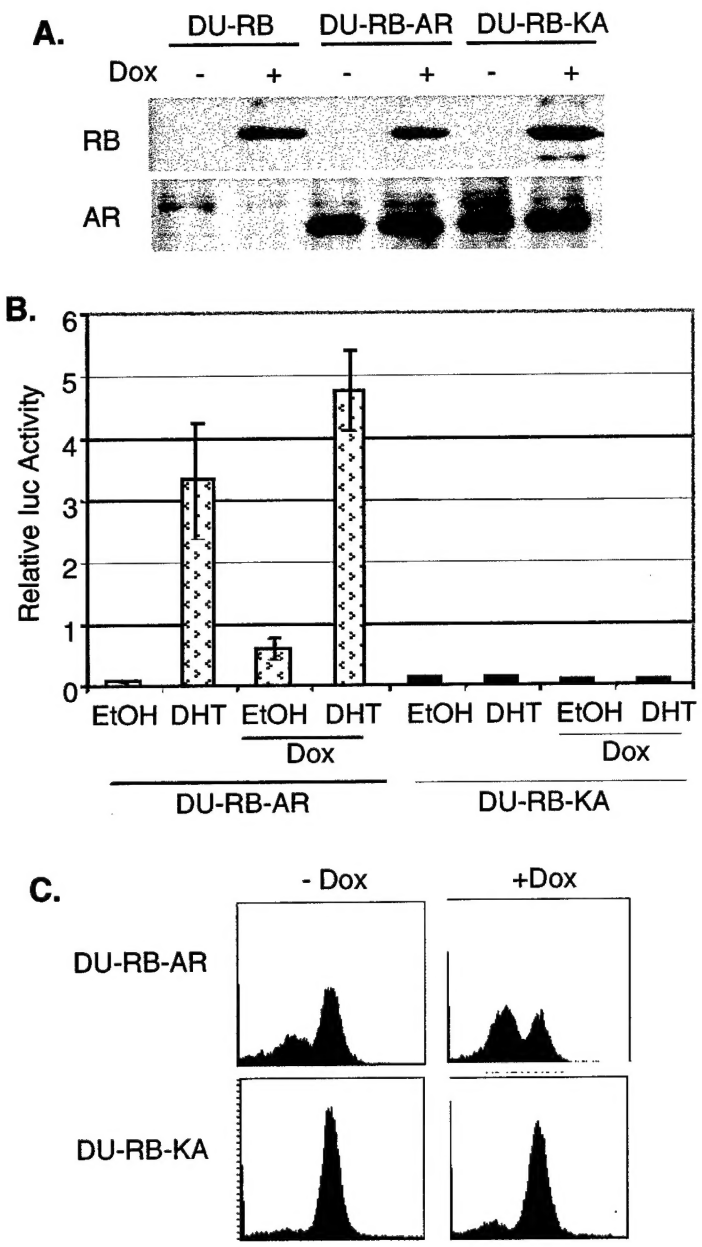

Figure 6. RB stimulated the AR's transactivation activity, which was required for the apoptosis. **A.** Western blotting of total cell extracts of DU-RB, DU-RB-AR, and DU-RB-KA (expressing AR-K630A mutant) cells with anti-RB and anti-AR antibodies as in Figure 1B. **B.** DU-RB-AR and DU-RB-KA cells were transfected with an AR reporter plasmid (MMTV-AR), and were treated with CDT media in the presence of DHT or the vehicle EtOH, as indicated. Dox, where indicated, was added to the cells for the last seven hours before harvest. Luciferase activity was measured and presented as relative units. Triplicate samples were analyzed for every data point and standard deviations displayed as error bars. The experiments were repeated three times with similar results. C. DU-RB-AR and DU-RB-KA cells were treated wth Dox in CDT media for twenty four hours, stained with DiOC6, and analyzed as in Figure 3C.

constitutive expression of the AR (DU-RB-AR cells without Dox) also did not show proliferative differences from the parental cells.

We then studied the effects of re-expressing RB together with the AR in DU-RB-AR cells. We observed a significant reduction in the numbers of attached cells 24 hrs after induction of RB expression (Figure 2A and B). This reduction in cell numbers was apparently not caused by a block to enter S phase as measured by BrdU incorporation (Figure 2C), but by cell death through apoptosis. As shown in Figure 3A, RB induction resulted in the appearance of Sub-G1 cell fractions in flow cytometry measuring cellular DNA content, and the externalization of phosphatidylserine (PS) on the plasma membrane as measured by Annexin V staining and flow cytometry (Figure 3B). Thus, the combined re-expression of RB and the AR created

an apoptotic activity that was not present when RB or the AR was re-expressed alone.

Apoptoic mechanisms can be generally divided into mitochondria dependent and independent groups. To determine the involvement of mitochondria in RB+AR induced apoptosis, we measured the mitochondria membrane integrity by $DiOC_6$ staining. $DiOC_6$ is selectively retained in the mitochondria when the mitochondria membrane potential is intact. Figure 3C shows that re-expression of RB led to the loss of mitochondria retention of $DiOC_6$. The extent of this loss is greater than those induced by chemicals that are widely used to induce mitochondria-mediated cell death such as etopside and okadaic acid.

We next determined the activation status of several key caspases by their cleavage and the cleavage of the caspase substrate PARP. As shown in Figure 4A, RB induction caused significant cleavage of PARP, caspase 3, 9, and 8. The extent of these effects were comparable to those induced by okadaic acid. The activation of caspases 9 and 3 are consistent with the well-established mitochondria death pathway (mitochondria damage>cytochrome C release>caspase 9 cleavage>and caspase 3 cleavage). The cleavage of caspase 8 could be mediated by other activated caspases (including caspases 9 and 3). Alternatively, caspase 8 cleavage could be due to the activation of the death receptor pathway or other mechanisms upstream of mitochondria. To determine the role of mitochondria in RB+AR-mediated apoptosis, we ectopically expressed Bcl-xl, an anti-apoptosis member of the Bcl-2 family, through an adenovirus vector. Bcl-xl protects mitochondria membrane damage and therefore blocks mitochondria-mediated apoptosis. We infected R5 cells with Ad-Bcl-xl 24 hours before RB induction. As shown in Figure 4B, adenovirus-mediated expression of Bcl-xl, but not the control LacZ, completely blocked cleavage of caspase 8, as well as caspases 9 and 3. Bcl-xl also completely prevented apoptosis (Figure 4C). We conclude from these results that RB+AR caused mitochondria damage to induce apoptosis.

RB activates AR-mediated transcription activation to induce apoptosis

Previous studied have demonstrated that RB and the AR could physically interact in vitro with purified RB and the AR proteins, and in vivo with mammalian two-hybrid type assay (Lu and Danielsen, 1998; Yeh et al., 1998). In the latter assay, the RB-AR interaction was determined to be 4 fold weaker than the interaction between the AR and its cofactors such as the ARA70. RB interacted with the N-terminal part of the AR and this interaction was independent on the AR ligand DHT. In AR reporter assays, it was shown that RB-AR interaction stimulated the AR transactivation activity in the presence of DHT (Lu and Danielsen, 1998; Yeh et al., 1998). With our inducible cells, we could now demonstrate in vivo interaction of RB and the AR through coimmunoprecipitation in cell extracts as shown in Figure 5A. There results suggest a direct relationship between RB and the AR.

RB and the AR both have well established biochemical activities. We investigated how RB and the AR affected each other's activities to gain insights into the mechanisms of RB+AR induced apoptosis. The best established function of RB is the repression of E2F. We determined the effects of RB induction on a E2F reporter in the absence (DU-RB cells) and presence (DU-RB-AR cells) of the AR. As shown in Figure 5B, induction of RB repressed the E2F reporter activity about 2 fold in both DU-RB and DU-RB-AR cells in the presence of the AR ligand DHT. When RB was expressed from transient transfection, repression of the E2F reporter was to a greater extent, likely due to the higher levels of RB protein in transiently transfected cells. When an unphosphorylable RB was expressed by transient transfection, it led to further greater repression of E2F, which suggested that the RB protein expressed in DU-145 cells was at least partially phosphorylated which could result in the release of E2F repression. Importantly again, transient transfection of RB also led to nearly identical degrees of E2F repression in DU-RB and DU-RB-AR cells, indicating that the co-expression of the AR did not alter RB's ability to repress E2F. Together with the BrdU labeling results in Figure 2C, these results also demonstrate that the effects

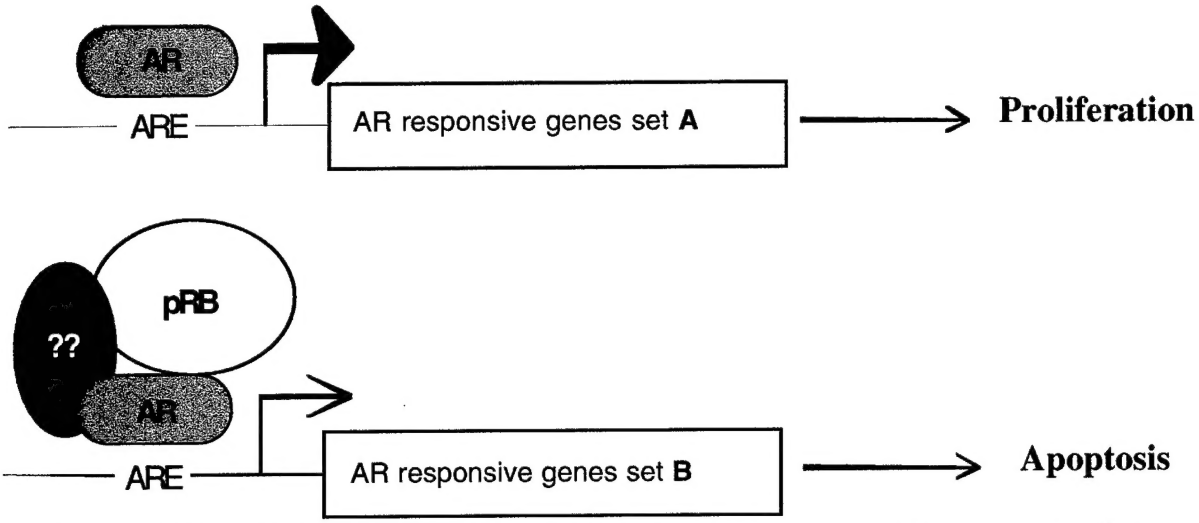

Figure 7. A working model for RB+AR induced apoptosis. Based on our results, we propose the following model for the functional relationship between RB and the AR. RB interacts with the AR to activate its transaction activity. The RB-mediated activation of the AR may lead to the expression of a set of AR responsive genes, whose products may function to induce apoptosis. Activation of AR by DHT in the absence of RB may lead to the expression of a different set of genes, whose products promote the already-established role of the AR in prostate cells proliferation and differentiation. Other factors may also be involved in this differential regulation of the AR.

of RB on DU-145 cells were very similar to a number of other RB-insensitive tumor cells as well as normal fibroblasts.

We measured the AR activity with the widely used AR-responsive MMTV-luc reporter. As shown in Figure 6B, the AR in DU-RB-AR cells responded to DHT with about 15-20-fold activation, which is similar to previous reports with transiently transfected AR in this cell line. Importantly, co-expression of RB, in the charcoal dextran treated (CDT) media without added DHT, significantly stimulated AR's transactivation activity in the same assay (about 5-fold). RB did not significant alter AR's transactivation activity in the presence of DHT. These results demonstrate that, when co-expressed, RB stimulate AR's transactivation activity.

We next determined whether RB could induce apoptosis of DU-RB-AR cells in the CDT media without DHT, where it could stimulate AR's transactivation activity. As measured by the $DiOC_6$ assay (Figure 6C), the extent of apoptosis was the same in CDT as compared with that in FBS (see Figure 3C). Thus, the activation of AR transactivation activity by RB in the CDT media was sufficient to induce apoptosis. The fact that greater AR transactivation activity induced by DHT could not induce apoptosis in the absence of re-expressed RB further demonstrated that the combined expression of RB and the AR resulted in a new apoptosis inducing activity, not just a enhancement of a weaker apoptotic activity of the AR.

We used a genetic approach to determine the role of AR's transactivation activity in the RB-AR induced apoptosis. It was recently demonstrated that a K630-to-A mutation at the accetylation consensus site abrogated AR's transactivation activity (Fu et al., 2002). We transfected the same DU-RB cell line with a pCDNA3-AR-KAzeo construct and selected with zeocin for clones with AR-KA expression, as we did for the DU-RB-AR cell line. A representative DU-RB-KA cell line was shown in Figure 6A. Expression levels of the AR-KA protein in this cell line were comparable with the levels of wild type AR protein expressed in the DU-RB-AR cell line, so were the levels of RB expression induced by Dox. As expected, the AR-KA in this cell line was unable to transactivate the MMTV-Luc reporter in response to androgen stimulation (Figure 6B). It also

failed to be stimulated by RB expression (Figure 6B). As shown in Figure 6C, expression of RB in this cell line did not induce mitochondria damage. We conclude that the AR's transactivation activity is required for RB-AR mediated apoptosis.

A working model

We have formed a working model to explain the experimental results obtained so far (Figure 7). The findings that potent activation of the AR by its ligand in the absence of RB does not induce apoptosis while less potent activation of the AR by RB effectively induces apoptosis, and this activation is required for apoptosis as demonstrated by the inability of the transactivation defective mutant AR, AR-KA, strongly suggest that AR-responsive genes activated by the AR in the absence of RB (set A in the Figure) are different from those activated in the presence, or by, RB (set B in the Figure). It is likely that other factors are also involved in the differential gene expression regulation of the AR by RB. Further studies will identify differentially activated cellular genes and how RB regulates the target gene spectrum of the AR.

Key Research Accomplishments

- Through combined restoration of RB and the AR in RB and AR mutant prostate cancer DU-145, we identified a novel, apoptosis-inducing activity of RB that is entirely dependent upon the transactivation activity of the AR.

Reportable Outcomes

A manuscript describing our findings as presented in this report is being submitted for publication.

Conclusions

Experiments proposed in Aim 2 of this grant have been successful in improving our understanding of the roles of the tumor suppressor pRB and the AR in prostate cancer cells. One functional role of the tumor suppresser pRB in prostate cells may be to promote cell death in an AR-dependent manner. Prostate cancer cells may gain a survival advantage when RB and/or the AR is mutated during the disease course. The requirement for both RB and the AR for apoptosis should have implications in prostate cancer gene therapy.

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